

TITLE OF THE INVENTION  
CLONING AND IDENTIFICATION OF THE MOTILIN RECEPTOR

CROSS-REFERENCE TO RELATED APPLICATIONS

5                   xxxxxx

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

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10   REFERENCE TO MICROFICHE APPENDIX

xxxxxx

FIELD OF THE INVENTION

15                   The present invention is directed to a novel human DNA  
sequence encoding a motilin receptor, the receptor encoded by the  
DNA, and the uses thereof.

BACKGROUND OF THE INVENTION

20                   Gastrointestinal (GI) motility is a coordinated neuromuscular  
process which transports nutrients through the digestive system.  
Impaired GI motility, can lead to irritable bowel syndrome, constipation  
and diabetic and post-surgical gastroparesis and is one of the largest  
health care burdens of industrialized nations. Motilin, a 22 amino acid  
25                   prokinetic peptide is expressed throughout the gastrointestinal tract in a  
number of species including humans. Released from endochromaffin  
cells of the small intestine, motilin exerts a profound effect on gastric  
motility with the induction of interdigestive (phase III) antrum and  
duodenal contractions. The unrelated macrolide antibiotic  
erythromycin also possesses prokinetic properties mediated by its  
30                   interaction with motilin receptors. These account for erythromycin's  
GI side-effects, including vomiting, nausea, diarrhea and abdominal  
muscular discomfort.

35                   Motilin receptors have been detected in the GI tract and recently  
in the central nervous system, but their molecular structure has not been  
reported. Although motilin receptor characterization has been actively  
pursued in humans and other species since the isolation of motilin from

porcine intestine in 1972, the receptor itself has not been isolated nor cloned.

Motilin is highly conserved across species and is synthesized as part of larger pre-prohormone. Mature 22 amino acid motilin is generated by removal of its secretory signal peptide and cleavage at the first C-terminally located dibasic prohormone convertase recognition site. Using radioligand binding, autoradiography and *in vitro* bioassays, high affinity and low density, motilin receptors were detected in smooth muscle cells of the gastrointestinal tract of humans, cats and rabbits. Cerebellar brain receptors for motilin were also described supporting the notion that motilin may act in the central nervous system. Native motilin receptors appear to be coupled to G proteins and activate the phospholipase C signal transduction pathway resulting in  $Ca^{2+}$  influx through L-type channels.

The development of safe and selective motilin receptor agonists is likely to aid the treatment of disorders resulting from impaired GI motility. Thus, it would be desirable to be able to isolate, and clone the motilin receptor, and to use this in assays for agonists and antagonists.

## SUMMARY OF THE INVENTION

The present invention is directed to a novel G-protein coupled receptor (GPCR), designated as motilin receptor. Two spliced forms of the motilin receptor were identified: MTL-R1A, which encodes a functional seven-transmembrane domain form, and MTL-R1B, which encodes a truncated five-transmembrane domain form. Both forms make up embodiments of this invention.

Another aspect of this invention are nucleic acids which encode the motilin receptor, which are isolated, or free from associated nucleic acids.

Other aspects of this invention include assays for identifying motilin ligands which are agonists and antagonists of a motilin receptor comprising contacting a candidate ligand with a motilin receptor and determining if binding occurred.

Another aspect of this invention is a method for determining whether a ligand is capable of binding to a motilin receptor comprising:

- (a) transfecting test cells with an expression vector encoding motilin receptor;
- (b) exposing the test cells to the ligand;
- (c) measuring the amount of binding of the ligand to the motilin receptor;
- (d) comparing the amount of binding of the ligand to the motilin receptor in the test cells with the amount of binding of the ligand to control cells that have not been transfected with the motilin receptor
- where if the amount of binding of the ligand to the test cells is greater than the amount of binding of the ligand to the control cells, then the substance is capable of binding to motilin receptor.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the DNA sequence of motilin receptor gene including 5' untranslated region (SEQ.ID.NO.:1). Intronic sequences are shown in lower case type.

Figure 2 shows the DNA sequence of motilin receptor spliced form A (MTL-R1A) (SEQ.ID.NO.:2).

Figure 3 shows deduced amino acid sequence of MTL-R1A (SEQ.ID.NO.:3).

Figure 4 shows the DNA sequence of motilin receptor spliced form B (MTL-R1B) (SEQ.ID.NO.:4).

Figure 5 shows the deduced amino acid sequence of MTL-R1B (SEQ.ID.NO.:5).

Figures 6 A-C compare DNA and protein sequence for MTL-R1A and MTL-R1B.

Figure 7 shows the DNA sequence of puffer fish clone 75E7 (SEQ.ID.NO.:6).

Figure 8 shows the deduced amino acid sequence of puffer fish clone 75E7 protein sequences (SEQ.ID.NO.:7).

Figure 9 shows the comparison of human MTL-R1A and puffer fish clone 75E7 protein sequences.

Figure 10 is a graph illustrating the pharmacological characterization of the cloned MTL-R1A in the aequorin bioluminescence assay in HEK-293 cells.

Figure 11 is a graph illustrating the pharmacological characterization of the cloned MTL-R1A in the [<sup>125</sup>I]-Tyr<sup>7</sup>-human motilin binding assay.

5 As used throughout the specification and claims, the following definitions apply:

“Substantially free from other proteins” means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other proteins. Thus, for example, a MTL-R1 protein  
10 preparation that is substantially free from other proteins will contain, as a percent of its total protein, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non- MTL-R1 proteins. Whether a given MTL-R1 protein preparation is substantially free from other proteins can be  
15 determined by such conventional techniques of assessing protein purity as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate detection methods, *e.g.*, silver staining or immunoblotting.

“Substantially free from other nucleic acids” means at least  
20 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other nucleic acids. Thus, for example, a MTL-R1 DNA preparation that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acid, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and  
25 even more preferably no more than 0.1%, of non- MTL-R1 nucleic acids. Whether a given MTL-R1 DNA preparation is substantially free from other nucleic acids can be determined by such conventional techniques of assessing nucleic acid purity as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*,  
30 ethidium bromide staining, or by sequencing.

“Functional equivalent” means a receptor which does not have the exact same amino acid sequence of a naturally occurring motilin receptor, due to alternative splicing, deletions, mutations, or additions, but retains at least 1%, preferably 10%, and more preferably  
35 25% of the biological activity of the naturally occurring receptor. Such derivatives will have a significant homology with a motilin receptor and

can be detected by reduced stringency hybridization with a DNA sequence obtained from a motilin receptor. The nucleic acid encoding a functional equivalent has at least about 50% homology at the nucleotide level to a naturally occurring receptor nucleic acid.

- 5           “Ligand” means any molecule which binds to a motilin receptor of this invention. These ligands can have either agonist, partial agonist, partial antagonist or antagonist activity.

## DETAILED DESCRIPTION OF THE INVENTION

- 10           The cloning of GPCR's related to the hypothalamic and pituitary receptor for the growth hormone (GH) secretagogues (GHSs) which mediate sustained pulsatile GH release has been recently described. (McKee *et. al.*, 1997 *Genomics* 46:426-434, which is hereby incorporated by reference). One of these clones, GPR38, possessed the  
15           most significant amino acid sequence identity to the human GHSR (52%) (rising to as high as 86% in transmembrane domains (TM). GPR38 was classified as an orphan GPCR (GPCRs for which a natural ligand has not been identified).

- GPR38 was isolated from a human genomic DNA library  
20           and contained a single intron of approximately 1 kb, as shown in FIGURE 1. cDNA clones were isolated to obtain the nucleotide sequence of correctly spliced GPR38 mRNA. Efforts to isolate cDNA clones by standard library screening proved unsuccessful.

- A combination of RACE and RT-PCR techniques resulted  
25           in the identification of two spliced forms for GPR38. These two GPR38 cDNAs use distinct splice donor sites and a common acceptor site (perfect match to consensus exon-intron splice acceptor junction sequence [pyrimidine-rich stretch ag/TG]). GPR38-A mRNA (imperfect match to consensus donor sequence [TGC/gt]) encodes a polypeptide of  
30           412 amino acids with seven alpha-helical TM domains, the hallmark feature of GPC-Rs, whereas GPR38-B encodes a 363 amino acid polypeptide with five TM domains (perfect donor sequence [CCG/gt]). Northern blot analysis failed to reveal an expression profile for GPR38. However, when RNase protection was employed expression was  
35           demonstrated in stomach, thyroid and bone marrow.

It accordance with this invention, it has been found that GPR38 is the motilin receptor. Thus, this invention is directed to the human motilin receptor, its functional equivalents, motilin receptors from other species which can be isolated using fragments of the human  
5 motilin DNA as probes, and to splice variants of the motilin receptor.

The intact motilin receptor of this invention was found to have structural features which are typical of G-protein linked receptors, including seven transmembrane (TM) domains, three intra- and extracellular loops, and the GPCR protein signature sequence. The TM  
10 domains and GPCR protein signature sequence are noted in the protein sequences of the GPCR in Figures 6A-C.

A high-throughput assay was developed which measures  $\text{Ca}^{2+}$  release with the bioluminescent  $\text{Ca}^{2+}$  sensitive-aequorin reporter protein (capable of measuring ligand-induced  $\text{IP}_3$ -coupled mobilization  
15 of intracellular calcium and concomitant calcium-induced aequorin bioluminescence). Expression of cloned GPR38-A in cell membranes was confirmed using epitope-tagged protein which revealed a single protein species with a molecular weight of approximately 45,000 daltons containing an open reading frame encoding 412 amino acids  
20 (SEQ. ID.NO.:3). The DNA and deduced amino acid sequence are given in SEQ.ID. NO.:2 and SEQ.ID. NO.:3, respectively.

A broad set of peptide and non-peptide molecules were tested at a single concentration in transiently transfected HEK-293/aeq17 cells (100 nM peptide, 10  $\mu\text{M}$  non-peptide). Significant bioluminescent  
25 responses were recorded for the peptide motilin and the non-peptide macrolide erythromycin, which was reported to be a competitive agonist at motilin receptors. Full dose-response curves confirmed this observation.

Nucleotide sequence analysis revealed two splice forms of  
30 human motilin receptor both of which make up further aspects of this invention. The first (MTL-R1A) encodes a seven transmembrane domain receptor. The full length open reading frame appears to contain 412 amino acids. The second splice form (MTL-R1B) diverges in its nucleotide sequence from MTL-R1A just before the predicted amino  
35 acid of the sixth transmembrane domain (TM6).

In the MTL-R1B, TM5 is truncated and fused to a contiguous reading frame of about 86 amino acids, followed by a translation stop codon. The DNA and amino acids sequences encoding MTL-R1A and MTL-R1B are given in FIGURES 2-5.

5 A further aspect of this invention is a related motilin receptor gene, evident in the teleost puffer fish *Spherooides nephelus*. Screening of a puffer fish genomic library identified a single clone (75E7) containing an open reading frame of 363 amino acids (approximately 54% identical at the protein level) which contains a  
10 similar exon-intron structure to GPR38. Analysis of clone 75E7 shows an amino acid sequence to contain 363 amino acids with a molecular weight of approximately 41,323 daltons. (FIGURE 8). DNA sequence of puffer fish clone 75E7 is given in SEQ.ID.NO.:6, and a comparison of human MTL-R1A and puffer fish clone 75E7 protein sequences is  
15 given in FIGURE 9.

Another aspect of this invention relates to vectors which comprise nucleic acids encoding a motilin receptor or a functional equivalent. These vectors may be comprised of DNA or RNA; for most cloning purposes DNA vectors are preferred. Typical vectors include  
20 plasmids, modified viruses, bacteriophage and cosmids, yeast artificial chromosomes and other forms of episomal or integrated DNA that encode a motilin receptor. It is well within the skill of the ordinary artisan to determine an appropriate vector for a particular gene transfer or other use.

25 A further aspect of this invention are host cells which are transformed with a gene which encodes a motilin receptor or a functional equivalent. The host cell may or may not naturally express a motilin receptor on the cell membrane. Preferably, once transformed, the host cells are able to express the motilin receptor or a functional  
30 equivalent on the cell membrane. Depending on the host cell, it may be desirable to adapt the DNA so that particular codons are used in order to optimize expression. Such adaptations are known in the art, and these nucleic acids are also included within the scope of this invention. Generally mammalian cell lines, such as HEK-293, COS, CHO, HeLa,  
35 NSJ, CV-1, GC, GH3 or VERO cells are preferred host cells, but other

cells and cell lines such as *Xenopus oocytes* or insect cells, may also be used.

Human embryonic kidney (HEK 293) cells and Chinese hamster ovary (CHO) cells are particularly suitable for expression of motilin receptor proteins because these cells express a large number of G-proteins. Thus, it is likely that at least one of these G-proteins will be able to functionally couple the signal generated by interaction of motilin receptors and their ligands, thus transmitting this signal to downstream effectors, eventually resulting in a measurable change in some assayable component, *e.g.*, cAMP level, expression of a reporter gene, hydrolysis of inositol lipids, or intracellular  $\text{Ca}^{2+}$  levels.

A variety of mammalian expression vectors can be used to express recombinant motilin in mammalian cells. Commercially available mammalian expression vectors which are suitable include, but are not limited to, pCR2.2 (Invitrogen), pMC1neo (Stratagene), pSG5 (Stratagene), pcDNA1 and pcDNA1amp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), and pSV2-dhfr (ATCC 37146). Following expression in recombinant cells, motilin receptors can be purified by conventional techniques to a level that is substantially free from other proteins.

The specificity of binding of compounds showing affinity for motilin receptors is shown by measuring the affinity of the compounds for recombinant cells expressing the cloned receptor or for membranes from these cells. Expression of the cloned receptor and screening for compounds that bind to motilin receptors or that inhibit the binding of a known, radiolabeled ligand of motilin receptors to these cells, or membranes prepared from these cells, provides an effective method for the rapid selection of compounds with high affinity for a motilin receptor. Such ligands need not necessarily be radiolabeled but can also be nonisotopic compounds that can be used to displace bound radiolabeled compounds or that can be used as activators in functional assays. Compounds identified by the above method are likely to be agonists or antagonists of



motilin receptors and may be peptides, proteins, or non-proteinaceous organic molecules.

Such molecules are useful in treating a variety of gastric conditions, including gastric motility disorders (intrinsic myopathies or neuropathy), functional defects, disorders which are secondary to neurologic disorders including spinal cord transections, amyloidosis, collagen vascular disease (e.g. scleroderma), paraneoplastic syndromes, radiation-induced dysmotility, diabetes, infections, stress-related motility disorders, psychogenic/functional disorders, other drugs which affect motility (e.g. beta adrenergic drugs which may delay gastric emptying, cholinergic agents or opiates, or serotonin receptor antagonists), gastroparesis (diabetic or post-surgical), gastro-esophageal reflux disease, constipation, chronic idiopathic pseudo-obstruction and acute fecal impaction, postoperative ileus, gallstones, infantile colic, preparation for colonoscopy and endoscopy, duodenal intubation, irritable bowel syndrome, non-ulcer dyspepsia, non-cardiac chest pain and diarrhea.

The pharmacological characterization of the cloned MTL-R1A in the aequorin bioluminescence assay in HEK-293 cells is shown in Figure 10 and in the [<sup>125</sup>I]-Tyr<sup>7</sup>-human motilin binding assay (Figure 11). Motilin at concentrations as high as 10  $\mu$ M gave no bioluminescent response above background levels in cells that were not transfected with the MTL-R1A cDNA expression vector. Similarly, non-transfected cells did not show appreciable binding of [<sup>125</sup>I]-Tyr<sup>7</sup>-human motilin.

The rank order of potency for motilin, motilin peptide fragments and non-peptide molecules is consistent with experiments performed on native motilin receptors, from stomach or intestinal tissues.

Due to the high degree of homology to GPCRs, the motilin receptor of this invention is believed to function similarly to GPCRs and have similar biological activity. They are useful in understanding the biological and physiological pathways involved in gastrointestinal motility. They may be also used to scan for motilin agonists and antagonists; as in particular to test the specificity of identified ligands.

The following, non-limiting Examples are presented to better illustrate the invention.

5

### EXAMPLE 1

Sequence Comparison of MTL-R1 (GPR38) to human GHS-R, Puffer Fish 75E7 and Identification of Alternatively Spliced Forms.

10 Inspection of the MTL-1 genomic DNA sequence revealed two potential mRNA splice sites corresponding to consensus boundaries for exon/intron junctions. An imperfect donor site (TGC/gt) was found at nucleotides 1929-31 (Fig. 1), a perfect donor site (CCG/gt) was found at nucleotides 2080-82, and a single perfect splice acceptor site (sequence  
15 [pyrimidine-rich stretch ag/TG]) was observed at nucleotides 2729-32. To determine which splice forms exist naturally, RACE (rapid amplification of cDNA ends) was performed on thyroid poly (A)+ mRNA and RT-PCR (reverse transcriptase polymerase chain reaction) was conducted on HEK-293/aeq17 cells transfected with the MTL-1  
20 genomic DNA construct. Directional RACE reactions were conducted on thyroid poly (A)+ mRNA that had previously been shown by RNase protection assay to contain transcripts for MTL-1R. Primer API 5'-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3' (SEQ.ID.NO.:8) corresponds to the 5' end of the coding region including the  
25 presumptive Met initiation codon located within the cloning vector. 5'RACE1 corresponds to the 3' end of the MTL-1R coding region including the translation termination codon TAA. 5' RACE1: 5'-TTA TCC CAT CGT CTT CAC GTT AGC GCT TGT CTC-3' (SEQ.ID.NO.:9).

30 RACE reactions were carried out on 1 µg of thyroid poly (A)+ mRNA using the Marathon cDNA amplification/advantage PCR kit as per the manufacturer's instructions (Clontech) using the following Touchdown PCR amplification conditions: 94°C for 1 min., 5 cycles of 94°C for 30 sec. and 72°C for 4 min.; 5 cycles of 94°C for 30 sec. and  
35 70°C for 4 min.; 25 cycles of 94°C for 20 sec and 68°C for 4 min. An approximately 1.4 kb amplified product was identified which hybridized

with a <sup>32</sup>P-labeled probe derived from the TM 2-4 region (3F/4R probe) of the MTL-R. This product was subcloned into PCR-Script vector (Invitrogen) and sequenced.

As diagrammed in Figures 6A-C, DNA sequence analysis  
5 revealed two distinct sequences corresponding to alternative use of two splice donor sequences and a common splice acceptor sequence. These results were confirmed by transfecting the MTL-1 genomic construct containing the complete ORF interrupted by a single intron of approximately 0.7 kb into HEK-293/aeq17 cells. mRNA was the  
10 isolated (Poly (A)<sup>+</sup> Pure Kit, Ambion) and shown by Northern blot analysis using the 3F/4R probe to give two hybridizing bands: 2.4 kb containing the unspliced intron and approximately 1.4 kb encoding spliced forms. RT-PCR was then performed (Superscript 2 One-Step Kit, Life Technologies) on MTL-1 mRNA from transfected HEK-  
15 293/aeq17 cells using the forward primer 5' RACE1 and reverse primer 3' RACE2 (TM5 region): 5'-CTG CCC TTT CTG TGC CTC AGC ATC CTC TAC-3' (SEQ.ID.NO.:10)

An approximately 500 bp product was cloned (TA vector pCR2.2, Invitrogen), sequenced and shown to be a mixture of both  
20 splice forms. Assembly of the complete ORF for MTL-1A without intronic sequence was performed by ligation of an exon 1 fragment (Not I digestion of a MTL-1 plasmid containing the intron in pCDNA-3) to pCDNA-3.1 containing a Not I/EcoRI exon 2 fragment.

To document protein expression, an MTL-1A plasmid encoding a  
25 amino-terminal FLAG epitope was constructed by ligation of a Pme I fragment from the MTL-1A/pCDNA-1.1 vector into the EcoRV site of pFLAG/CMV-2 vector (Kodak Imaging Systems). Following transfection of this plasmid into HEK-293/aeq17 cells, a protein of the expected size (approximately 48 kDa) was detected in crude cell  
30 membranes by immunoblot analysis.

## EXAMPLE 2

### Identification of Ligand Specific to Motilin Receptor

35 To identify a ligand for this orphan GPCR and to determine whether the full length, 7 TM domain GPR38-A is a functional GPCR, a

high-throughput assay was developed which measures  $\text{Ca}^{2+}$  release with the bioluminescent  $\text{Ca}^{2+}$  sensitive aequorin reporter protein (capable of measuring ligand-induced  $\text{IP}_3$ -coupled mobilization of intracellular calcium and concomitant calcium-induced aequorin bioluminescence).

- 5 Expression of GPR38-A in cell membranes was confirmed using epitope-tagged protein which revealed a single protein species with a molecular weight of approximately 45,000 daltons.

A broad set of peptide and non-peptide molecules was tested at a single concentration in transiently transfected HEK-293/aeq17 cells (100  
10 nM peptide, 10  $\mu\text{M}$  non-peptide). Significant bioluminescent responses ( $> 4$ -fold over background) were recorded for the peptide motilin and the non-peptide macrolide erythromycin, which was reported to be a competitive agonist at motilin receptors. Full dose-response curves confirmed this observation. The half-maximal effective concentration  
15 ( $\text{EC}_{50}$ ) for human/porcine motilin was  $2.1 \pm 0.5$  nM motilin whereas erythromycin was considerably less potent ( $2000 \pm 210$  nM; as expected from studies performed on native motilin receptors).

The signal transduction pathway for the cloned GPR38-A motilin receptor (MTL-R1A) is through activation of phospholipase C, which  
20 has been reported for native motilin receptors. Direct radioligand binding studies with [ $^{125}\text{I}$ ] human motilin on cell membranes prepared from transfected cells show that MTL-R1A confers high affinity and specific binding ( $K_d = 0.1$  nM;  $B_{\text{max}} = 240$  fmol/mg protein) which are strongly G protein coupled ( $> 80\%$  inhibition of binding with 100 nM  
25  $\text{GTP}\gamma\text{S}$ ).

### EXAMPLE 3

#### Functional Activation of the MTL-1A Receptor

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The aequorin bioluminescence assay is a reliable test for identifying G protein-coupled receptors which couple through the  $\text{G}\alpha$  protein subunit family consisting of  $\text{G}_q$  and  $\text{G}_{11}$  which leads to the activation of phospholipase C, mobilization of intracellular calcium and  
35 activation of protein kinase C. Measurement of MTL-1A expression in the aequorin-expressing stable reporter cell line 293-AEQ17 (Button,

D. et. al.,1993 *Cell Calcium* 14: p. 663-671.) was performed using a Luminoskan RT luminometer (Labsystems Inc., Gaithersburg, MD).

293-AEQ17 cells (8 x 10<sup>5</sup> cells plated 18 hrs. before transfection in a T75 flask) were transfected with 22 µg of human MTL-R1A  
5 plasmid DNA: 264 µg lipofectamine. Following approximately 40 hours of expression the apo-aequorin in the cells was charged for 4 hours with coelenterazine (10 µM) under reducing conditions (300 µM reduced glutathione) in ECB buffer (140 mM NaCl, 20 mM KCl, 20 mM HEPES-NaOH [pH=7.4], 5 mM glucose, 1 mM MgCl<sub>2</sub>, 1 mM  
10 CaCl<sub>2</sub>, 0.1 mg/ml bovine serum albumin). The cells were harvested, washed once in ECB medium and resuspended to 500,000 cells/ml. 100 µl of cell suspension (corresponding to 5x10<sup>4</sup> cells) was then injected into the test plate, and the integrated light emission was recorded over 30 seconds, in 0.5 second units. 20 µL of lysis buffer (0.1% final  
15 Triton X-100 concentration) was then injected and the integrated light emission recorded over 10 seconds, in 0.5 second units. The "fractional response" values for each well were calculated by taking the ratio of the integrated response to the initial challenge to the total integrated luminescence including the Triton X-100 lysis response.

20

#### EXAMPLE 4

Binding of [<sup>125</sup>I] Human Motilin to Crude Membranes from HEK-293 Cells transfected with the MTL-R1A cDNA.

25 The binding of [<sup>125</sup>I] human motilin to crude membranes prepared from HEK-293/aeq17 cell transfectants was performed as follows. Crude cell membranes were prepared on ice, 48 hrs. post-transfection. Each T-75 flask was washed twice with 10 ml of PBS, once with 1 ml homogenization buffer (50 mM Tris-HCl [pH 7.4], 10  
30 mM MgCl<sub>2</sub>. 10 ml of homogenization buffer was added to each flask, cells were removed by scraping and then homogenized using a Polytron device (Brinkmann, Syosset, NY; 3 bursts of 10 sec. at setting 4). The homogenate was centrifuged for 20 min. at 11,000 x g at 0°C and the resulting crude membrane pellet (chiefly containing cell membranes and  
35 nuclei) was resuspended in homogenization buffer supplemented with 1.5 % BSA (0.5 ml T75 flask) and kept on ice.

Binding reactions were performed at 20°C for 1 hr. in a total volume of 0.5 ml containing: 0.1 ml of membrane suspension (approximately 1 µg protein), 10 µl of <sup>125</sup>I-human motilin, 10 µl of competing drug and 380-390 µl of homogenization buffer. Bound radioligand was separated by rapid vacuum filtration (Brandel 48-well cell harvester) through GF/C filters pretreated for 1 hr. with 0.5% polyethylenimine. After application of the membrane suspension to the filter, the filters were washed 3 times with 3 ml each of ice-cold 50 mM Tris-HCl [pH 7.4], 10 mM MgCl<sub>2</sub>, and the bound radioactivity on the filters was quantitated by gamma counting. Specific binding (> 90% of total) is defined as the difference between total binding and non-specific binding conducted in the presence of 100 nM unlabeled human motilin. Competition binding data were analyzed by a nonlinear curve-fitting program (Prism V, version 2.0; GraphPad Software, San Diego, CA). Results shown are the means (+/- SEM) of triplicate determinations; Human motilin was radiolabeled with <sup>125</sup>I at <sup>7</sup>Tyr to a specific activity of approximately 2000 Ci/mmol (Woods Assay, Portland, OR).

Structure-function analysis suggest that the motilin peptide minimally contains an N-terminal region (amino acids 1-7) essential for activity, linked to a C-terminal alpha helical domain which stabilizes the N-terminal active site region activity. The rank order of potency of several motilin peptide analogs in the MTL1-A functional and binding assays correlates with their reported potency measured by *in vitro* contractility assays (Table 1) performed on native motilin receptors in intestinal tissue. These results are summarized in Table 1 below.

Ligand	Cloned MTL-1A Receptor (human)	
	Aequorin Assay (EC <sub>50</sub> nM)	[ <sup>125</sup> I] hmotilin binding (IC <sub>50</sub> ,nM)
human motilin (MTL)	2.1	0.5
erythromycin	2000	427
roxithromycin	1950	613
metoclopramide	>10,000	>10,000
cisapride	>10,000	>10,000

canine motilin	4.4	0.2
Leu13 MTL	3.9	0.2
1-11 MTL	138	127
1-12 MTL	72	14
1-13 MTL	3.8	0.9
1-19 MTL	4.1	0.3
10-22 MTL	>10,000	1100

The unrelated prokinetic agents metoclopramide and cisapride which have affinity for dopamine and/or 5-HT receptors were inactive, even at high (10  $\mu$ M) doses.

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#### EXAMPLE 5 Southern Blot Analysis

A genomic Southern blot (EcoRI and BamHI-digested DNA, 10  $\mu$ g/lane) was hybridized with the ORF of MTL-1A. Post-hybridizational washing stringencies were at 55°C 4 X SSPE after which the filters were dried and exposed to X-ray film for 5 days at -70°C. Lambda Hind III DNA markers were (in kb), 23.1, 9.4, 6.6, 4.4, 2.3, 2.1. Southern blot analysis conducted in a variety of mammalian and non-mammalian species revealed a simple hybridization pattern consistent with a single, conserved gene encoding MTL-1A.

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#### EXAMPLE 6 Puffer Fish Clone 75E7

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Screening of a puffer fish genomic library identified a single clone (75E7) containing an open reading frame of 363 amino acids with approximately 54% protein sequence identity to the human MTL-R1A. In addition, 75E7 has a similar intron-exon structure to the human MTL-R1A. 75E7 may be the ortholog of the human MTL-R1A.

25

EXAMPLE 7  
Expression of the MTL-1A Gene

5           Transcripts of MTL-1A were detected by RNase Protection Assay (RPA). Synthesis of high-specific activity radiolabeled antisense probes and the RPA was conducted using a kit (MAXIscript and HybSpeed RPA kits; Ambion, Austin, TX) essentially as described by the manufacturer. The anti-sense cRNA MTL-1A probe was  
10 synthesized from a cDNA template encompassing nt 1234 to 1516 of the human MTL-1A inserted behind the T7 promoter in pLitmus 28 (New England Biolabs, Beverly, MA). Digestion of the construct with Stu I generated a cRNA transcript approximately 340 nt in size with approximately 60 nt of vector sequence. Input poly A<sup>+</sup> mRNA  
15 (Clontech, Palo Alto, CA) was 5 g for the MTL-1A probe and 0.1 µg for a control human actin probe. Precipitated fragments were subjected to slab-gel electrophoresis (42 cm x 32 cm x 0.4 mm) in 5 % acrylamide/Tris-borate-EDTA buffer containing 8 M urea. The gels were fixed, dried and autoradiographed on film (X-Omat; Kodak,  
20 Rochester, NY) for 1-3 days (MTL-1A) or 2 hrs. (actin).

          The distribution profile of MTL-1A mRNA was examined in a panel of GI and non-GI human tissues. MTL-1A mRNA could be detected in whole stomach (most prominently), thyroid, and bone marrow but was absent from several brain regions and other non-CNS  
25 tissues.